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OXIDIZING ENZYMES IN BRAIN
EXTRACTS

A DISSERTATION SUBMITTED TO THE
FACULTY OF THE DIVISION OF THE
BIOLOGICAL SCIENCES IN CANDIDACY FOR
THE DEGREE OF DOCTOR OF PHILOSOPHY

DEPARTMENT OF PHYSIOLOGY

1935

By
MABEL BLAKE COHEN

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Reprinted from
AMERICAN JOURNAL OF PHYSIOLOGY
Vol. 119, No. 1, May, 1937



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THE UNIVERSITY OF LEIDEN

OXIDIZING ENZYMES IN BRAIN CATALYSTS

A DISSERTATION, IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY, SUBMITTED BY
J. H. VAN DER KAM
TO THE FACULTY OF SCIENCE, UNIVERSITY OF LEIDEN

1924

LEIDEN, 1924



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LEIDEN, 1924

OXIDIZING ENZYMES IN BRAIN EXTRACTS¹

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Received for publication December 10, 1936

The physiological and pathological activity of the central nervous system is dependent in high degree on its metabolic processes and so on its enzyme complement. Attempts at analysis, however, result in considerable confusion, due to the complexity of organization and to local metabolic differences in the whole organ. A simpler system, more favorable for study though less complete, results from minimizing structural differences and destroying some enzyme systems. This has been achieved by preparing a homogeneous colloidal suspension of brain in water. Such a sol has a small residual respiration, limited apparently by available substrate, and contains many of the enzymes present in whole brain, although others, particularly those involved in carbohydrate oxidation, are inactivated. The sol, and a protein-lipoid fraction obtained from it by isoelectric precipitation, carry concentrated systems for oxidizing succinic acid and paraphenylenediamine. A partial analysis of the enzyme mechanisms present is described below.

METHODS. A rabbit was stunned by a blow, the brain quickly removed, pia and blood peeled off, and the desired tissue weighed and kept on ice. Only cerebral cortex and cerebellum were used, since the underlying tissues contain a large proportion of white matter which is difficult to cytolyze. When cool, the tissue was minced, then ground, and finally dispersed with nine volumes of iced distilled water. The suspension was centrifuged 20 minutes and the supernatant solution pipetted off. About $\frac{3}{4}$ of the total volume was used. This supernatant colloidal solution (referred to in subsequent pages as "the sol") was seen under the microscope to be cell-free and virtually structureless except for dispersed particles, although it was cloudy in bulk. Further, filtration through a porcelain candle had little effect on its properties. No material settled out on standing.

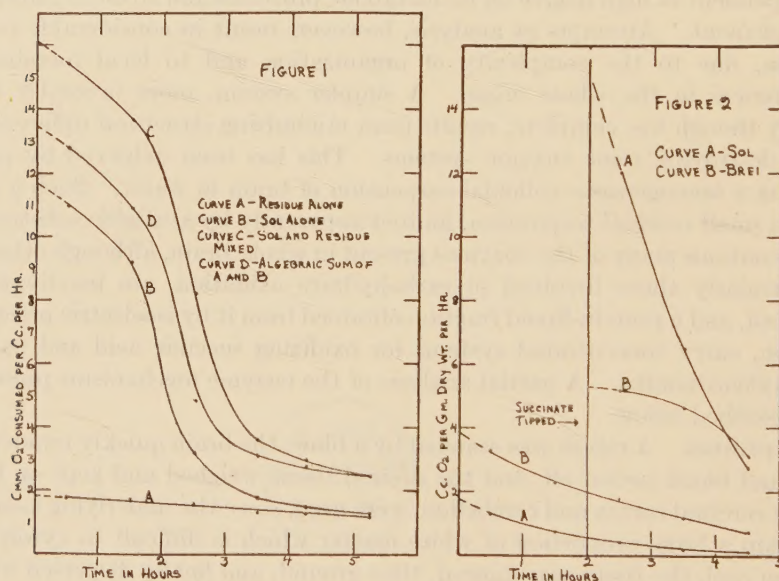
Other preparations were also used: 1, the washed residue obtained on centrifugation, containing mostly cell debris and white matter; 2, a protein-lipoid fraction prepared from the solution by isoelectric precipitation (at

¹ Aided in part by a grant from the Otho S. A. Sprague Foundation. A preliminary report of this material appeared in this Journal **109**: 21, 1934.

about pH 4.6), washing and resolution in dilute alkali; and 3, dehydrated or dehydrated and defatted preparations of the precipitate.

Oxygen consumption was measured in modified Warburg manometers (Gerard, 1931) at 37°C. The total volume of fluid in each chamber was always 1.0 cc., 0.1 cc. of this being N/2 NaOH and the rest either brain extract or extract plus the desired reagent. The preparations were unbuffered unless otherwise stated.

RESULTS. I. *Respiration of the sol.* A. *Residual respiration* of the sol was comparatively high the first hour, but fell gradually to nil by the 4th to 6th, presumably with the exhaustion of available substrate. Typical Q_{O_2} values (cubic millimeters oxygen consumed per cubic centimeter brain



solution per hour) are shown in figure 1. The total oxygen consumption for the first three hours averaged 18.1 in all experiments (50). Since each preparation was homogeneous, duplicate manometers gave excellent agreement, but rates for different brain sols varied from 15 to 30. The 3 hour average per milligram dry weight was 0.35 to 0.5 mm. O₂ per hour (compare with 3 to 5 for minced brain), that for the first hour being 1.0. For intact brain (Brookens, Ectors and Gerard, 1936) initial values per milligram dry weight may be as high as 20, though in vitro average values are 5 to 10 during the first hour.

This residual respiration falls as the sol ages, and even at 1 to 2°C. disappears in 1 to 2 weeks though 85 per cent remains after 3 days. The respiration is partly heat stable, over $\frac{1}{3}$ remaining after a half-hour at 60°C.; lactate oxidation is likewise partially heat stable (see later).

The R.Q. of the sol is 0.48 (compare with an R.Q. of 1.0 for whole brain, Dickens, 1936). Such a low value could not be associated with the complete oxidation of carbohydrate, protein or lipoid. It suggests, rather, that dehydrogenations are not carried to completion, so that little CO_2 results.

What substrate is oxidized during residual respiration, which is quite constant from one brain to another, is of interest; the more so since it is not one of the carbohydrate derivatives responsible for the major part of the respiration of intact brain (e.g., Holmes, 1932). Sodium moniodoacetate inhibits glucose oxidation between the triose-phosphate and pyruvic acid stages (Meyerhof, 1933) or even earlier and should, therefore, decrease residual respiration to the extent that glucose or hexose-phosphate is involved. The observed depression, with M/100 iodoacetate, is only 10 per cent (compared with 87 per cent in brain slices), so that no considerable carbohydrate oxidation is occurring. Further, lactate added to sol is oxidized, but glucose hardly at all. According to Quastel and Wheatley (1932), many narcotics inhibit the dehydrogenation of glucose, lactate and pyruvate by brain tissue, yet urethane in the concentration they used (0.25 per cent) gave no significant inhibition and M/100 sodium arsenite, another dehydrogenase inhibitor, decreased the oxygen consumption of the sol only 6 per cent (1 expt.), while that of brain brei was decreased by 73 per cent.

Cyanide, by contrast, cuts out two-thirds (63 per cent) of the residual respiration, presumably by action on the indophenol oxidase. This is true for M/100 cyanide; a more dilute solution, M/1000, increases oxidations by as much as 100 per cent in some experiments, 31 per cent on the average. Apparently at sufficient dilution cyanide accelerates certain oxidations more than it inhibits others. Cyanide-hematin is a powerful catalyst for lipoid oxidations (Wright and Van Alstyne, 1931) and cyanide increases the oxidation of methyl-glyoxal (Meyerhof, 1925; Ariyama, 1928; see below). It prevents the inhibitory action of pyruvic acid on lactate oxidation (Green and Brosteaux, 1936) and may itself undergo a coupled oxidation with glucose in alkaline solution (Harned and Deere, 1934). The accelerating action of cyanide on the respiration of *Chlorella* in the resting state and its inhibiting effect on the extra respiration in nutrient solutions will be recalled (Emerson, 1927). An attempt to demonstrate oxidation of sol lipoids in the presence of cyanide by a change in iodine number was unsuccessful.

To eliminate completely larger structural elements, the sol was filtered through a porcelain candle. This decreases oxygen consumption at most by one-fifth, and even this loss may have been due to a rise in temperature during filtration. The R.Q. and substrate oxidizing ability of the filtered material (so far as tested) are unchanged. The enzyme reactions of the sol are, therefore, essentially independent of organized structure. The

indophenol oxidase and probably other enzymes are associated with fairly large particles since they can be centrifuged out of solution with even moderately high speeds (Ogston and Green, 1935b; Hoerr, personal communication); and for brain sol also sufficient centrifuging reduces both the residual respiration and the oxidation of added succinate and paraphenylenediamine. The particles in this case must be under 0.02 to 0.03μ since little or no enzyme loss resulted from filtration through porcelain of such pore size.

The washed residue from the original centrifugation, with almost no respiration of its own, may still double the respiration of the sol on remixing with it. The average increase is 20 per cent (fig. 1) for unfiltered sol, 50 per cent after filtration, and the excess oxygen consumption is over in four hours. On remixing the washed residue with its washings there was no such effect. The accelerating influence of the debris is not related to its contributing "structure" to the mixture since no increase of sol respiration results from addition of inert colloids, such as acacia or albumin. Of course a specific active adsorption compound might be formed from moieties in the separate fractions; and it is interesting that urethane (0.25 per cent), which presumably acts by adsorption, entirely abolishes the extra respiration of mixed sol and residue while not affecting their separate respirations.

The extra respiration of the mixture is partly due to the oxidation of additional substrate, for not only is the rate greater but the total oxygen consumed, dependent on available substrate, is also increased by 20 per cent. It is striking that added lactate likewise increases the respiration of the mixture one-fourth more than it increases the sum of the respirations of sol and residue treated separately. Further factors are important, however, since mixing solution and residue after the oxygen consumption of each has ceased does not restore the respiration of the mixture as it should if additional substance can be burned by it.

B. *The effect of pH.* The pH of the solution immediately after preparation ranges from 6.7 to 7.0 from one brain to another, and is maintained during several days on ice, or throughout an experiment at 37°C . Residual respiration is not sensitive to moderate pH changes, for a series of 20 preliminary experiments showed this to be greatest at pH 7.6 and only 6 to 8 per cent less at pH 7.2 or 8.0. Addition of 1/10 volume of M/15 phosphate buffer at pH 7.4 does not noticeably affect the residual respiration of the sol or its oxidation of any substrate except methyl glyoxal. It may, moreover, be acidified to pH 3.8 and reneutralized without significant decrease of oxygen consumption.

C. *Oxidation of substrates.* The rate (and amount) of oxygen consumption of the sol might be limited by the amount of enzyme or of substrate present. In the latter case, addition of an appropriate substrate should

increase respiration. The following were tried in M/100 and sometimes M/1000 final concentration: glucose, fructose, sodium lactate, glycerophosphate, pyruvate, succinate, citrate and oleate, methyl glyoxal, glycine and paraphenylenediamine. Of these, sodium succinate, sodium glycerophosphate, sodium lactate, fructose, methyl glyoxal and paraphenylenediamine are oxidized. Sodium oleate and citrate inhibit the residual respiration by 30 to 50 per cent. Results are summarized in table 1.

Glucose. In a large series of experiments, no increased oxygen consumption could be demonstrated on adding M/100 or M/1000 glucose. An essential enzyme seems peculiarly sensitive to destruction of cell structure, for glucose addition increases the respiration of chopped but not cytolysed brain about 40 per cent. Progressive dilution of brain brei with

TABLE 1
Effect of substrates on oxygen consumption of the solution

SUBSTRATE ADDED	NUMBER OF EXPERIMENTS	O ₂ CONSUMPTION IN PER CENT OF SOL ALONE	
		<i>first hour</i>	<i>average first 3 hours</i>
Glucose, M/100	10	93	94
Lactate, M/100	5	178	150
Citrate, M/100	2	70	74
Oleate, M/100	4	47	54
Oleate, M/100 + CaCl ₂ , M/100	2	75	71
Fructose, M/100	2	125	129
Pyruvate, M/100	2	110	95
P-phenylenediamine, M/100	2	1450	1220
Succinate, M/100	5	2150	1570
Methyl glyoxal, M/100, unbuffered	4	115	111
Methyl glyoxal, M/100, buffered	2	129	125
Glycerophosphate, M/100	2	162	136

water does not stop glucose oxidation until a dilution (1:14) greater than that in the sol is reached (table 2), presumably because the inner cells of a clump are protected against cytolysis. Brei with water added, which oxidizes glucose, and the uncentrifuged suspension, which does not, differ as to the degree of cytolysis and the amount of dilution of cell protoplasm consequent to cytolysis. The former factor seems the more important since, when complete cytolysis is assured by thoroughly grinding the chopped brain, a dilution of less than 1:2 entirely eliminates glucose oxidation. This can hardly entail a serious loss of coenzyme, and active glucose dehydrogenases have been obtained from liver (Harrison, 1931) and blood cells (Warburg and Christian, 1931) after destroying cell structure. Further, a loss of the oxidase-cytochrome system, if involved at all, is not important, since methylene blue does not restore glucose oxidation.

Presumably the dehydrogenase has somehow been inactivated by our procedure of water cytolysis.

Other workers have noted that dilution per se may cause diminution of respiration. Thus Warburg (1911) found that a concentrated suspension of laked red cells uses 60 to 75 per cent more oxygen than intact cells, but that dilution causes a rapid decrease in oxygen usage, and Krebs (1935) confirmed this for a suspension of kidney brei. He suggested that systems which lose activity on dilution involve reactions of ternary or higher orders, since dilution rapidly decreases the chance of multiple collision. In the present system, except with glucose, dilution has little effect, so either binary reactions are involved or more than one reactant is carried on the same colloidal particle. This seems to be the case for the oxidase-cytochrome-succinic-dehydrase system.

Lactate is rapidly oxidized by the sol, the extra oxygen consumption (80 per cent) being nearly completed in an hour. It is also oxidized by

TABLE 2
Effect of water dilution on oxidation of glucose by brain brei

BREI	WATER	RINGER	Q _{O₂} WITHOUT GLUCOSE	Q _{O₂} WITH GLUCOSE	PER CENT INCREASE
<i>gram.</i>	<i>cc.</i>	<i>cc.</i>			
0.05	0	0.75	403	623	43
0.05	0.2	0.55	444	577	30
0.05	0.3	0.45	438	680	55
0.05	0.4	0.35	452	483	7
0.05	0.5	0.25	389	452	16
0.05	0.6	0.15	299	325	9
0.05	0.7	0.05	166	203	22

the washed residue, increasing respiration about 200 per cent. Added to a mixture of sol and residue, lactate causes an extra respiration greater than the sum of the separate increases. Thus, with lactate, the oxygen consumption of sol and residue run separately totals 22.2 cmm. for the first hour, while that of the mixture is 28.4, an increase of 28 per cent. Recombination of dehydrogenase and coenzyme, partially separated in residue and sol, could account for this increase.

The oxidation of lactate is not entirely abolished by heating, even to boiling. The oxygen uptake of heated sol plus lactate (13.5 cmm.) is 3 times that of the heated sol alone (4.5 cmm.) and over half that of the unheated sol plus lactate (20.2 cmm.). What the heat stable mechanism for lactate oxidation may be is unknown—no such effect was noted with any other substrate.

Methyl-glyoxal is oxidized to a moderate extent, the average increase in respiration being 11 per cent for 3 hours. As it oxidizes, acid is formed,

sufficient to precipitate the sol proteins after 1 to 2 hours. Addition of buffer prevents this precipitation, and oxygen consumption is then increased by 25 per cent. The comparatively small amount of oxygen consumed by the methyl-glyoxal (25 cmm. or $1\mu\text{M}$) would not correspond to enough acid production to precipitate the proteins, which requires about $8\mu\text{M}$ of HCl. Probably, therefore, methyl-glyoxal is undergoing a reaction involving acid production without oxygen consumption, as in dismutation to lactic acid.

Sodium succinate and p-phenylenediamine increase the residual respiration markedly, 20 and 14 times respectively for the first hour after addition. Succinate is oxidized so vigorously that, in M/100 concentration, oxidation

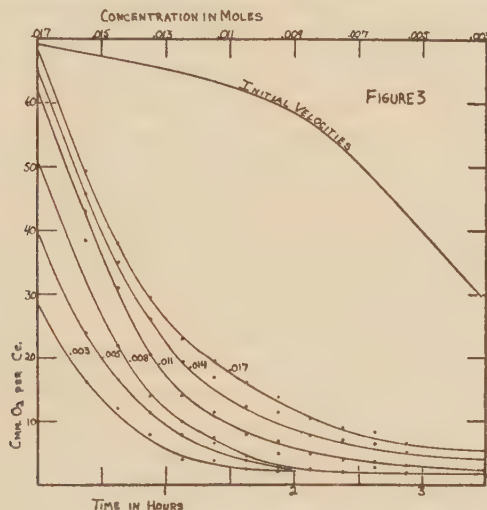


Fig. 3. Oxygen consumption of the sol in the presence of succinate in 0.003 to 0.017 M. concentration. Top curve represents initial oxygen consumption rates plotted against molar concentration.

is ended in 2 to 3 hours. Upon addition of more substrate, the oxygen consumption resumes nearly its original high rate. These substrates are also oxidized (though more slowly) by a sol that has been kept in the ice-box until its residual respiration is zero.

Although the oxidation by tissues and tissue extracts of succinate and p-phenylenediamine are commonly associated quantitatively (Thunberg, 1930; Quastel and Wheatley, 1932), the enzyme systems involved are not identical. Thus, Quastel and Wheatley (1931) have found that malonate inhibits the oxidation of succinate but not of p-phenylenediamine by brain slices, presumably by competing for the succinic dehydrogenase. Similarly, in the present brain sol malonate inhibited succinate oxidation, reducing to $\frac{1}{2}$ or $\frac{1}{3}$ the extra oxygen consumption due to succinate alone,

but had no effect on that of p-phenylenediamine. P-phenylenediamine is supposed to be oxidized by the Atmungsferment directly, while succinate oxidation depends on this plus a special dehydrogenase at least. Results with cyanide and methylene blue (see below) also point in this direction. Ogston and Green (1935a) offer evidence that the cytochrome-indophenol oxidase system catalyzes the reaction of succinic acid and its dehydrogenase with molecular oxygen and that even for p-phenylenediamine oxidation cytochrome is required in addition to the oxidase.

The respiration of chopped brain (50 mgm.) is increased 3 to 4 times in the first hour after adding sodium succinate (0.1 cc. M/10); that of the sol (1 cc.) 20 times (fig. 2). Succinate oxidation by the sol is almost complete in 2 hours, when 111 cmm. O_2 have been consumed, while in the same time the brei has consumed only 98 cmm. The extra oxygen used (above the normal respiration without substrate) per milligram dry weight of chopped brain and of sol respectively is 6.8 and 17.8 cc. for the first 2 hours; and the sol excess is even more striking for the first 30 minutes, during which the amounts are 2.4 for brei, 6.7 for sol. The sol is able, therefore, per unit dry weight, to oxidize succinate almost three times as rapidly as the brain from which it was prepared. Whether this represents a true concentration of enzyme, better diffusion relations in the sol, or loss of inhibitors present in whole brain (a "sparing" action of lactate has been reported, Quastel and Wheatley, 1932), has not been established.

At 37°C. and 750 mm. Hg, 125 cmm. of oxygen should oxidize 0.1 cc. M/10 sodium succinate to fumarate. In some experiments, however, the actual oxygen consumed exceeded this by as much as 60 to 65 cmm. Therefore, some of the oxygen consumed is used either to further oxidize the fumarate, or is being diverted to another substrate (vide St. Gyorgyi's theory of the carrier rôle of succinate-fumarate). The oxygen consumption never reached 250 cmm. (one molecule of oxygen per molecule of succinate). The R.Q. for the oxidation of succinate is zero—a slight CO_2 production (7 cmm. CO_2 to 192 cmm. O_2) is due to the residual respiration itself—and, of course, the transformation of succinate to fumarate does not involve the liberation of CO_2 . The other oxidations, by the excess oxygen not accounted for by this reaction, must also stop before CO_2 is formed.

Oxidation rates were measured at succinate concentrations from 0.0009 M to 0.11 M in an effort to determine a velocity constant. The curves obtained have roughly the same slope between 0.003 M and 0.017 M (see fig. 3) but, assuming either one molecule or one atom of oxygen is used per molecule of succinate, calculations do not yield a constant.

Glycerophosphate is not oxidized as rapidly as the above substrates, but increases sol oxygen consumption 62 per cent for the first hour, 36 per cent for the first three. Green (1936) has found the glycerophosphate de-

hydrase more concentrated in brain than in other tissues. Its activity is enhanced by cyanide and sodazide, depressed by narcotics, and the main oxidation product is glyceraldehydephosphate.

Sodium citrate and *oleate* inhibit respiration, possibly by removing calcium ion; and addition of equivalent amounts of CaCl_2 partially restores oxidations. Calcium ion thus seems to influence the residual oxidations of brain sol in the reverse direction to oxidations in peripheral nerve (Chang, Shaffer and Gerard, 1935) or brain slices (Dickens and Greville, 1935).

D. *Effect of cyanide.* The respiration of sol alone is inhibited by M/100 and accelerated by M/1000 cyanide, but the extra oxygen consumption in the presence of substrates is uniformly inhibited. Methyl-glyoxal is an exception, its oxidation by sol or by chopped brain being accelerated 4 to 5 times (table 3). A similar influence of cyanide on methylglyoxal oxidation

TABLE 3
Effect of cyanide on the oxidation of substrates by the sol

SUBSTRATE ADDED	NUMBER OF EXPERIMENTS	OXYGEN CONSUMPTION IN PER CENT*
None.....	5	37
M/100 methyl glyoxal.....	2	529
M/100 Na succinate.....	3	10
M/100 Na succinate + 0.01 per cent methylene blue.....	3	18
M/100 p-phenylenediamine.....	2	4
M/100 p-phenylenediamine + 0.01 per cent methylene blue.....	2	3

* Values expressed in per cent of the oxygen consumption of the sol plus substrate without cyanide.

was reported by Meyerhof (1925) and confirmed by Ariyama (1928), who found the glyoxal was converted quantitatively to an unidentified volatile acid. Brain sol, however, almost doubles the oxygen consumption of a cyanide and glyoxal mixture, so that enzyme activity is also enhanced.

The oxidation of succinate is inhibited almost completely (92 per cent) by M/100 cyanide, and methylene blue (0.01 per cent) is able to restore it to one-fifth of normal. P-phenylenediamine oxidation, on the other hand, is completely inhibited by cyanide, and no restoration can be obtained with methylene blue. These results offer further evidence of two different oxidizing systems. Succinic acid presumably requires activation by a dehydrogenase as well as the presence of oxidized cytochrome before being oxidized, while p-phenylenediamine requires no special activation in the presence of the oxidase system (Keilin, 1929). Cyanide inhibits the oxidation of both compounds by preventing the oxidation of cytochrome. Methylene blue substitutes for the cyanide inhibited oxidase system and

completes the oxidation of succinate activated by its dehydrogenase (Gerard, 1932). Active succinate has a lower redox potential (-0.437 , pH 7) than the p-phenylenediamine molecule (estimated to be about $+0.36$) so that methylene blue, whose potential is $+0.011$, can oxidize only the former.

II. *Respiration of the centrifuge residue.* This residue, about $\frac{1}{3}$ by volume of the original suspension and consisting of a semi-solid mass of cell debris, membranes, blood vessels, uncytolized nerve fibers, etc., consumes oxygen 2 to 3 times as rapidly as does the sol. The rate falls to zero after 3 or 4 washings, but the washed residue is still able to oxidize succinate and p-phenylenediamine rapidly, lactate to a lesser extent (18 cmm. O_2 per cc. in 3 hours) and glucose not at all. With succinate, 128 cmm. O_2 per cc. are consumed by the residue during the first hour, which is more than that for an equal volume of sol but considerably less per mgm. dry weight (12 cc. for sol; 3.5 for residue). This is true also for the oxidation of p-phenylenediamine.

III. *The isoelectric precipitate.* When acid is added to the sol, a precipitate of protein and lipid components forms. It is most abundant at pH 4.6 and is partially redissolved in more acid or alkaline media. When redissolved in water and neutralized to litmus, this material still has a residual oxygen consumption $\frac{1}{2}$ to $\frac{2}{3}$ that of the original sol.²

The redissolved precipitate oxidizes succinate and p-phenylenediamine as rapidly as does the original sol, whereas the supernatant solution does not consume oxygen spontaneously or in the presence of any substrate tried. All activity, therefore, is in the precipitate. The decrease in residual respiration is not due to acid destruction of the enzyme, since if the solution is brought to pH 3.8 and reneutralized without separating it into fractions no decrease results. Substrate loss into the supernatant solution when the enzyme is precipitated probably accounts for the diminution.

The residual respiration of the precipitate is greatest on precipitation between pH 5.5 and 4.3. Below 4.3, although the volume of precipitate is still large, respiration is much smaller, and above 5.5 little precipitate is formed. The residual activity can be decreased by repeatedly washing the precipitate with acidified water or, less readily, with acidified phosphate solution, though the volume of the precipitate is little diminished. Two washings with water or three with phosphate are sufficient to abolish nearly all residual oxygen consumption.

Since succinate is oxidized by the precipitate, per milligram dry weight,

² It is worth noting that neutral red inhibits this respiration completely. A long series of experiments in which this dye was used as an indicator was thereby invalidated. It was later found that neutral red also inhibits the respiration of the sol, as well as the excess oxygen consumption produced by the addition of succinate. We are indebted to Dr. D. R. Briggs for first preparing the isoelectric precipitate.

more rapidly than by the solution, concentration of the succinate enzyme by fractional acid precipitation was attempted. Successive fractions were precipitated by small increments of acid and separately removed. The most active fraction (though not the greatest in bulk), obtained at pH 4.6 to 5.0, oxidizes succinate 2 to 3 times as rapidly as the original sol and 6 to 8 times as rapidly as brain brei. The purification of the enzyme is being pursued.

DISCUSSION. When brain is cytolyzed and extracted with a large volume of water, the solid debris continues to consume oxygen, indicating retention of substrates and catalytic systems. After washing, this disappears due to substrate loss, since added lactate, succinate and p-phenylenediamine are still oxidized. The catalysts are retained, including the soluble coenzyme for lactate oxidation. Probably some cells have not been fully cytolyzed even by this drastic handling.

The colloidal tissue extract also contains complete oxidation systems for, at least, lactate, methyl glyoxal, α glycerophosphate, succinate and p-phenylenediamine, and continues to use oxygen until substrate is gone. The residual respiration is not inhibited by moderate concentrations of cyanide, though it is by M/100 cyanide, nor by narcotics or arsenite, nor by iodoacetate—though respiration of intact brain is depressed by all these agents in the same concentrations. Glucose can hardly be the substrate, since the glucose dehydrogenase system is quickly inactivated by cell destruction and dilution, since iodoacetate does not inhibit, and since, finally, glucose is probably all changed to lactic acid during the preparation of the brain fractions. Succinate oxidation should be inhibited by arsenite and urethane, and it is quite possible that stronger concentrations of these agents, as of cyanide, would inhibit, for intact cells are commonly more sensitive to inhibitors than are their oxidizing systems when reconstructed *in vitro*. But added succinate is so rapidly oxidized that any originally present could hardly account for the feeble prolonged residual respiration. This most likely depends on lactate, which is present in significant amounts and which is slowly oxidized when added. Further, on remixing residue and sol the oxygen consumption exceeds the sum of the two separately and the same excess is found for the increased respiration due to lactate addition. Whether this excess is due to remixing dehydrogenase and coenzyme, largely separated by extraction, or improved concentration relations of the reactants (e.g., if most of the dehydrogenase remains behind while most of the lactate and carrier are extracted) we cannot say. Structural factors do not seem to be concerned.

The lactate oxidation is, further, incomplete ($R.Q. = 0.5$) and somewhat anomalous in that it is in part thermostable (again like the residual respiration). This is being further explored.

The ready oxidation of added succinate demonstrates the presence of

this dehydrogenase and the indophenol oxidase-cytochrome system. Oxidation of succinate yields no CO_2 , yet considerably more oxygen is used than required to change it all to fumarate. This extra oxygen could not all be accounted for by other substrates reducing fumaric (or oxalacetic) acid, as in Szent-Gyorgyi's (1936) scheme, since the total residual oxygen consumption (20 cmm. O_2) of the sol, which is a measure of oxidizable substrate, is only $\frac{1}{3}$ the amount in question. Further, when the extra oxygen consumption following one succinate addition is ended, and any substrate depending on it as a carrier fully oxidized, a second succinate addition again leads to greater oxygen use than theoretical. Malonate, which inhibits the succinic dehydrogenase, has no effect on the residual oxygen consumption of the solution, and finally, the total amount and rate of oxygen consumption when succinate and lactate are simultaneously or successively added is no greater than their separate sum.³ In this system, therefore, there is no evidence for a carrier rôle of succinate. (Compare Greville, 1936.) The additional oxygen usage presumably serves to carry oxidations past the fumarate stage.

Because of these non-stoichiometric relations attempts to analyze velocities and orders of reaction have had little success. The initial oxidation rate is independent of the concentration of succinate added over a considerable range. Yet the rate rapidly falls with time even when a large amount of succinate is initially present. Here the quantity left after a period of oxidation is still greater than that originally added in other experiments and which gave a maximal initial rate. An entirely analogous situation was encountered in the oxidation of lactate by *Sarcina lutea* (Gerard, 1930). Possibly substrate is relatively rapidly modified by one reaction to an intermediate form from which it is supplied to the catalytic system for oxidation at a rate dependent on the concentration of the intermediate.

SUMMARY

Rabbit brain was cytolized by grinding and suspending in 10 volumes of water and extracts made as follows: 1, the supernatant solution after centrifugation; 2, the washed centrifuge residue; 3, a preparation made from the sol by isoelectric precipitation, centrifuging and redissolving in dilute alkali. The oxidizing systems of these extracts were studied.

³ In 6 experiments on rat brain sol, paralleled by 2 on rabbit, the initial rate in sol alone was 18 during a half hour. With nothing tipped, the rates for the following 10 minutes and one hour were 14 and 11; with succinate tipped, 35 and 22; with lactate tipped, 24 and 12; and with both, 56 and 25. The increase with both substrates for the hour is equal to the sum of the increases produced by each alone: (25-11) compared to [(22-11) + (12-11)]. For the first 10 minutes the total rate together is the sum of the separate rates, but the increase above sol alone is greater in the mixture. A slight carrier action may therefore be present.

All three respire to a small extent in the absence of substrate, the oxygen consumption of the sol being about one-tenth that of the brain from which it was prepared. Addition of the centrifuge residue to the sol produces a coenzyme-like effect on residual respiration, the total oxygen consumption of the two together being 20 per cent greater than the sum of their separate ones. The factor in the residue is heat stable, and some part of the system is inactivated by urethane.

The sol oxidizes methyl glyoxal, glycerophosphate, lactate, fructose and, with great vigor, succinate and paraphenylenediamine. The activity of the succinate oxidizing system is 2 to 3 times as great as that of intact brain. Szent-Gyorgyi's suggested carrier rôle for the succinate-fumarate system is not confirmed on this extract.

The glucose oxidizing system is destroyed (or inactivated) by cytolysis. The lactate system is not completely destroyed, but its components are separated by centrifuging, so that lactate oxidation by sol or residue alone is less than by the mixture.

M/1000 cyanide stimulates and M/100 cyanide inhibits the residual respiration of the sol. It also stimulates the oxidation of methyl glyoxal, partly itself exerting a catalytic action and partly increasing the action of the enzyme. Cyanide inhibits the oxidation of succinate and paraphenylenediamine by the sol, and methylene blue partially removes the inhibition of succinate but not of paraphenylenediamine.

The extract prepared by isoelectric precipitation has at least two enzyme systems still present, those involved in succinate and paraphenylenediamine oxidation. This extract, per unit dry weight, contains 6 to 8 times as much succinate oxidizing activity as the original brain, indicating a partial purification of the enzyme.

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